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Identification of a Universally Primed-PCR-Derived Sequence-Characterized Amplified Region Marker for an Antagonistic Strain of *Clonostachys rosea* and Development of a Strain-Specific PCR Detection Assay

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We developed a PCR detection method that selectively recognizes a single biological control agent and demonstrated that universally primed PCR (UP-PCR) can identify strain-specific markers. Antagonistic strains of *Clonostachys rosea* (syn. *Gliocladium roseum*) were screened by UP-PCR, and a strain-specific marker was identified for strain GR5. No significant sequence homology was found between this marker and any other sequences in the databases. Southern blot analysis of the PCR product revealed that the marker represented a single-copy sequence specific for strain GR5. The marker was converted into a sequence-characterized amplified region (SCAR), and a specific PCR primer pair was designed. Eighty-two strains, isolated primarily from Danish soils, and 31 soil samples, originating from different localities, were tested, and this specificity was confirmed. Two strains responded to the SCAR primers under suboptimal PCR conditions, and the amplified sequences from these strains were similar, but not identical, to the GR5 marker. Soil assays in which total DNA was extracted from GR5-infested and noninoculated field soils showed that the SCAR primers could detect GR5 in a pool of mixed DNA and that no other soil microorganisms present contained sequences amplified by the primers. The assay developed will be useful for monitoring biological control agents released into natural field soil.

Clonostachys rosea Schroers, Samuels, Seifert & Gams (syn. *Gliocladium roseum* Bain.) is a common saprophyte in soil worldwide (10, 22, 25). It is antagonistic to other fungi, including important plant pathogens, in soil and plant material (11, 12, 24, 25, 29, 31). The mode of action responsible for this antagonism is not well understood, but mycoparasitism, substrate competition, antibiosis, and induced resistance all may play a role (25). Several strains of *C. rosea*, including GR5, with antagonistic activities against seed-borne diseases of cereals (12) were analyzed by universally primed PCR (UP-PCR) and found to be genetically very similar but not clonal (6). The release of biocontrol agents into the environment has created a demand for methods for monitoring and distinguishing them from indigenous strains. Monitoring a selected *Clonostachys* strain in soil using dilution plates has the disadvantage that it is not possible to distinguish the released strain from indigenous strains of the same species (13). Therefore, methods with high degrees of specificity and sensitivity are needed for detection of individual strains.

PCR has become an attractive tool for detection of specific microorganisms in microbial ecology, and much effort has been devoted to the development of primers that recognize specific species (8, 16, 19, 30). Genetically modified microorganisms can be recognized by PCR because the foreign sequence they carry differs from the background, thus allowing the design of primers that selectively recognize the target gene (3, 27, 28). PCR also can be used to recognize individual wild-type strains if unique sequences can be identified (1, 8, 19). Thus, PCR techniques also may be used to analyze environmental samples without culturing the microorganisms.

The UP-PCR technique (7) is similar to the randomly amplified polymorphic DNA (RAPD) technique (32), but longer primers (approximately 16 to 21 nucleotides) with unique designs are used (6, 8). The reactions are carried out at relatively high annealing temperatures and result in highly reproducible amplification products (fingerprints) from single organisms.

Our objective in this study was to use UP-PCR to develop a PCR detection method for a single *C. rosea* strain (GR5) from natural field soil. GR5 has promise as a biocontrol agent of *F. culmorum* in barley, based on in planta bioassays (unpublished results). By converting one of the UP-PCR-derived markers into a sequence-characterized amplification region (SCAR), we developed a simple PCR procedure for direct detection of GR5 released into field soil.

MATERIALS AND METHODS

Fungal strains, growth conditions, and DNA extraction. Strains of *C. rosea* and *Gliocladium* spp. (Table 1) were stored at −80°C in 10% glycerol and grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.). Fresh fungal mycelium (4 to 7 days old) of each isolate was scraped off petri dishes and added to one-third of the conical part of a 1.5-ml microcentrifuge tube. Total DNA was extracted basically as described by Bulat et al. (6), by adding 600 μl of buffer (50 mM Tris-HCl [pH 7.8]), 50 mM EDTA, 150 mM NaCl, 2.5% *N*-lauroyl sarcosine, 500 mM 2-mercaptoethanol, 600 μg of proteinase K [Sigma Chemical Co., St. Louis, Mo.] per ml) at 65°C for 4 h with frequent mixing. The NaCl concentration of the solution was then adjusted to 1 M, and two extractions to denature the proteins were carried out as follows. First, an equal amount of phenol-chloroform mixture (1:1) was added, left for 15 min at room temperature (20 to 25°C), and centrifuged at 12,000 × *g* for 2 min, and the aqueous phase was transferred to a new tube. An equal volume of chloroform-octanol mixture (24:1) was then

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TABLE 1. *C. rosea* and *Gliocladium* strains

Strain(s)	Designation in culture collections	Species	Origin
GR1[a]	IBT7519	*C. rosea*	Soil, Slagelse, DK[b]
GR2[a]	IBT7659	*G. solani*[c]	Potato, DK
GR3[a]	IBT7839	*C. rosea*	Soil, Annecy, France
GR4	IBT97905	*C. rosea*	Soil, Slagelse, DK
GR5	IBT97906	*C. rosea*	Soil, Taastrup, DK
GR6	IBT97907	*C. rosea*	Soil, Taastrup, DK
GR7	IBT97908	*C. rosea*	Soil, Taastrup, DK
GR8	IBT9354	*C. rosea*	Straw (soil), Slagelse, DK
GR9	IBT9366	*C. rosea*	Straw (soil), Slagelse, DK
GR10	IBT9367	*C. rosea*	Straw (soil), Slagelse, DK
GR11	IBT9368	*C. rosea*	Straw (soil), Slagelse, DK
GR12	IBT9369	*C. rosea*	Straw (soil), Slagelse, DK
GR13	IBT9370	*C. rosea*	Straw (soil), Slagelse, DK
IK726	IBT9371	*C. rosea*	Root of barley, Taastrup, DK
NS33[d]		*C. rosea*	Soil, United States
NS34[d]		*C. rosea*	Soil, United States
NS35[d]		*C. rosea*	Soil, United States
NS36[d]		*C. rosea*	Soil, United States
CTR 71-15[d]		*C. rosea*[e]	Cecropia, Jamaica
CTR 72-130[d]		*C. rosea*[e]	Bark, Venezuela
GJS 83-230[d]		*C. rosea*[e]	Root of tree, New Zealand
GJS 89-34[d,f]		*C. rosea*[e]	Bark of dead tree, Guyana
GJS 94-122[d]		*C. rosea*[e]	Dead yucca leaves, Mexico
B13[g]		*C. rosea*	New Zealand
B14[g]	ICMP 5254	*C. rosea*	New Zealand
B15[g]		*C. rosea*	New Zealand
B16[g]	CMI 192798	*C. rosea*	United Kingdom
	CBS 271.514	*C. rosea*	Seedling of pinus, The Netherlands
C 600[h]	CBS 224.72C	*C. rosea*	Wheat field soil, Germany
C 601[h]	CBS 224.72D	*C. rosea*	Wheat field soil, Germany
	CBS 710.864	*C. rosea*	Sclerotium of *Sclerotinia minor*, The Netherlands
5-95[h]		*C. rosea*	Soil, The Netherlands
C 684[h]	CBS 221.72B	*G. catenulatum*	Wheat field soil, Germany
	CBS 299.684	*G. catenulatum*	Berlin, Germany
Gliomix[i]		*G. catenulatum*	Finland
HJS 171[h]	CBS 702.97	*G. solani*	Rotten fruit, France
GR37, -38, -39, -40, -41, -42, -43, -44		*C. rosea*	Soil, Taastrup, locality 113, DK
GR45, -46, -47, -48,[f] -49, -50, -80		*C. rosea*	Soil, Flakkebjerg, locality 93, DK
GR51, -52, -53, -54, -55, -56, -57, -58, -59, -60, -61		*C. rosea*	Soil, Sjaelland Odde, locality 8, DK
GR62, -63, -64, -65, -66		*C. rosea*	Soil, Sjaelland Odde, locality 19, DK
GR67, -68, -69, -70		*C. rosea*	Soil, Sjaelland Odde, locality 165, DK
GR72, -73		*C. rosea*	Soil, Taastrup, DK
GR74, -75, -76, -77, -78, -79, -80, -81, -82		*C. rosea*	Soil, Aagerup, DK

[a] Provided by Ulf Thrane, Danish Technical University, Lyngby, Denmark.
[b] DK, Denmark.
[c] Classified by Hans Josef Schroers, CBS, Baarn, The Netherlands.
[d] Provided by Gary Samuels, Agricultural Research Service, Beltsville, Md.
[e] Anamorph of *Bionectria ochroleuca*.
[f] Strain that generates the diagnostic PCR product under nonoptimal conditions.
[g] Provided by Alison Stewart, Lincoln University, Canterbury, New Zealand.
[h] Provided by Hans Josef Schroers.
[i] Gliomix is the active ingredient in a commercial product of the same name (Kemira-OY, Helsinki, Finland).

added and left for 15 min at room temperature. After centrifugation at 12,000 × *g* for 2 min, the aqueous phase again was transferred, and the DNA was precipitated with isopropyl alcohol (0.6 volume) for a few minutes at 21°C. The precipitate was rinsed once with 70% ethanol, vacuum dried, and dissolved in TE buffer (1 mM Tris-HCl [pH 7.8], 0.1 mM EDTA). The final DNA concentration was 50 to 100 mg/ml. The DNA quality and optimal concentration for PCR for each strain were adjusted by testing dilution series in UP-PCR using a single UP primer.

UP-PCR amplification. The UP primers (Table 2) were used individually and in pairwise combinations (a total of 21 combinations). UP-PCR was performed as described by Bulat et al. (6) and Lübeck et al. (18) in a 20-μl volume containing 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 2.8 mM $MgCl_2$, 40 ng of primers, between 10 and 100 ng of total DNA, and 2 to 3 U of *Tsp* (*Thermus* sp.) DNA polymerase (courtesy of O. K. Kaboev, Petersburg Nuclear Physics Institute, St. Petersburg, Russia). The temperature optimum for *Tsp* polymerase is 69°C.

Alternatively, UP-PCR was performed in a 20-μl volume containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 0.4 mM dNTPs, 3.5 mM $MgCl_2$, 40 ng of primers, between 10 and 100 ng of total DNA, and 1 U of DynaZyme version 2.0 polymerase (Finnzymes OY, Espoo, Finland). The temperature optimum for DynaZyme is 72°C.

We used a high-ramping TC-1000M thermal cycler (Petersburg Nuclear Physics Institute) with 0.5-ml narrow tubes (Lenmedpolimer, St. Petersburg, Russia). PCR conditions were as follows (the times include temperature-ramping periods): 30 cycles with DNA denaturation at 92°C for 50 s (first denaturation step at 94°C for 2.5 min), primer annealing at 52 to 55°C for 90 s, and primer extension at 69°C for 60 s. The rate of ramping between the different temperatures was about 4°C/s. A final extension was performed at 69°C for 3 min.

We also performed UP-PCRs in a model PTC-150 MiniCycler (MJ Research, Watertown, Mass.) with standard 0.5-ml PCR tubes (Biozym Diagnostik GmBH, Oldendorf, Germany). PCR conditions were as follows (the times exclude temperature-ramping periods): 30 cycles with DNA denaturation at 92°C for 50 s

TABLE 2. Primers used in this study

Primer type	Primer	Sequence	Reference
UP	3-2	5′-TAAGGGCGGTGCCAGT-3′	9
	AS4	5′-TGTGGGCGCTCGACAC-3′	17
	AS15	5′-GGCTAAGCGGTCGTTAC-3′	9
	AS15inv	5′-CATTGCTGGCGAATCGG-3′	
	AA2M2	5′-CTGCGACCCAGAGCGG-3′	17
	Fok1[a]	5′-GGATGACCCACCTCCTAC-3′	17
	L21	5′-GGATCCGAGGGTGGCGGTTCT-3′	20
SCAR	GR5f	5′-TGGTTTCGGGCGGAGAGTA-3′	
	GR5r	5′-CCTCATGCCCTTCTGATTCA-3′	

[a] Arbitrary primer.

(first denaturation step at 94°C for 3 min), primer annealing at 53 to 56°C for 70 s, and primer extension at 72°C for 60 s. The ramping rate was about 2.4°C/s. A final extension was performed at 72°C for 3 min.

Amplification products were initially resolved electrophoretically in 1.7% agarose gels at 300 V for 30 to 40 min. Products from some of the amplifications also were resolved electrophoretically in 20-cm-long 5.5% polyacrylamide gels at 170 V for 10 h with cold Tris-borate-EDTA buffer.

Elution of PCR products, Southern blot hybridization, and sequencing. The DNA band of interest was cut from an acrylamide gel and placed in a 1.5-ml microcentrifuge tube. The gel slice was crushed with a pestle directly in the tube, and the tube was placed in boiling water for 7 min. The resulting DNA solution was reamplified using the UP primers AA2M2 and AS15inv (Table 2) but with 13 instead of 30 cycles. The PCR products were extracted with chloroform-octanol (24:1) and precipitated with 0.7 volume of isopropanol for 10 to 30 min at room temperature. The pellet was washed with 70% ethanol, dried, and redissolved in TE buffer. The resulting DNA was then ready to be sequenced.

For Southern blot hybridization, genomic DNA was digested with *Hin*dIII (Fermentas AB, Vilnius, Lithuania) and electrophoresed in a 0.7% agarose gel. The DNA was blotted onto a nylon filter (Hybond N, Amersham Pharmacia Biotech Ltd., Buckinghamshire, United Kingdom), and labeling and hybridization were carried out as described by Bulat et al. (6).

The PCR product was sequenced in both directions using the two primers AA2M2 and AS15inv and the ABI Prism Big Dye Sequencing System (Perkin-Elmer Applied Biosystems, Foster City, Calif.). The sequence for the marker had no homology with known DNA sequences as determined by using FASTA (21) and BLAST (2) searches to screen EMBL/GenBank. The sequences for GR5 and two closely related strains (GR47 and GJ 98-34) were deposited in GenBank.

Design of SCAR primers and PCR amplification conditions. From the sequence data, a pair of SCAR primers (GR5f and GR5r) with the expected size of the diagnostic product of 121 bp was designed (Table 2). Genomic DNA was amplified in PCR mixtures containing 0.6 pmol of GR5f, 1.4 pmol of GR5r, 20 mM Tris-HCl (pH 8.8), 2.8 mM $MgCl_2$, 10 mM KCl, 10 mM $(NH_4)SO_4$, 0.1% Triton X-100, 0.2 mM dNTPs, and 0.2 U of DynaZyme polymerase in a volume of 20 μl. The amplification conditions were as follows: 30 cycles with DNA denaturation at 92°C for 50 s (first denaturation step at 94°C for 3 min), primer annealing at 58°C for 80 s, and primer extension at 72°C for 40 s. The final extension step was performed at 72°C for 3 min. DNA extracted from soil samples was tested in dilution series using the SCAR primer pair, and 1/10 of the amplification products was checked on 1.7% agarose gels.

Extraction and purification of DNA from soil microorganisms. DNA was extracted from 0.5 g of soil by mixing with a lysing buffer (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 2.5% *N*-lauroyl sarcosine, 0.5 M 2-mercaptoethanol, 200 μg of proteinase K [Sigma] ml^{-1}) and incubated for 2 to 4 h at 65°C. The soil particles were precipitated by centrifugation for 1 min at 10,000 × *g*. This procedure was repeated twice by adding new lysing buffer to the pelleted soil particles. The supernatants from the extractions were combined, the NaCl concentration of the solution was adjusted to 1 M, and the solution was then extracted twice, first with a phenol-chloroform (1:1) mixture and then with chloroform-octanol (24:1). The DNA was precipitated with 0.6 volume of isopropyl alcohol. The pellet was rinsed with 70% ethanol, dried, and dissolved in 300 μl of Tris buffer (10 mM Tris-HCl, pH 8.0) at 50°C for 15 min. The crude DNA solution was purified twice with the Wizard DNA Clean-Up System (Promega Co., Madison, Wis.) according to the manufacturer's instructions and eluted with 50 μl of Tris buffer (10 mM Tris-HCl, pH 8.0).

As a positive control, each DNA sample was amplified in different concentrations with primers that recognize the ITS1 region of fungal nuclear ribosomal DNA. This control was performed to ensure the quality of the extracted DNA, to test for presence of fungal DNA, and to optimize the DNA concentration to minimize the risk of obtaining a false-negative result. The amplification conditions for ITS1 amplification using the primer pair X and Y were as described by Bulat et al. (6). DNA extraction for some of the samples had to be repeated until they fulfilled this criterion (out of 33 soil samples, 31 were used) (Table 3). Subsequently, DNA from each soil sample was tested with the SCAR primer pair for presence of the GR5-specific sequence.

Inoculation of *C. rosea* strain GR5 into field soil. The soil used for this experiment was field soil from two Danish barley fields (B23DK and B24DK) (Table 3). The B24DK soil sample was a composite sample from nine collections in the field. To avoid large soil aggregates and to obtain homogeneous material, the soil was sieved through a 4-mm sieve prior to inoculation with GR5 germinated conidia. A conidial suspension of GR5 (10^8 cells/ml) was added to 20 ml of potato dextrose broth (Difco) in an 100-ml Erlenmeyer flask and incubated overnight at 25°C on an orbital shaker at 180 rpm. The germinated conidia and young hyphae were harvested on a polyester filter membrane (42-μm mesh) and washed with sterile water. Two different amounts of the germinated conidia and hyphae (1 and 0.1 ml) were used to inoculate 10 g of soil in 50-ml capped plastic tubes. DNA from the inoculated soil was extracted and tested as described above.

Dilution plating for test of indigenous *C. rosea*. We tested for the presence of indigenous *C. rosea* in two of the noninoculated soil samples (B23DK and B24DK) by dilution plating of three replicate subsamples of soil (equal to 10 g [dry weight]). Each sample was homogenized with 90 ml of sterile water, and dilutions were plated on V8 agar (20% Campbell V8 juice, 2% Bacto agar [Difco], and 0.21% Triton X-100). The medium was adjusted to pH 7 and after autoclaving was amended with antibiotics (chloramphenicol [0.5 g/liter] and tetracycline [0.25 g/liter]). Isolates were identified in a stereomicroscope. *C. rosea* isolates were recultivated on potato dextrose agar amended with antibiotics and stored at −80°C in 10% glycerol until use.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences from strains GR5, GR47, and GJ 98-34 are AF141913, AF141914, and AF141915, respectively.

RESULTS

Identification of strain-specific UP-PCR markers. Seven UP primers, individually and in pairwise combinations (21 primer combinations), were tested for the ability to distinguish 14 *C. rosea* strains (Table 1). The primer combination AS15inv-AA2M2 amplified a unique 250-bp UP-PCR fragment from GR5 (Fig. 1). When used as a probe in Southern blots, this fragment was found as a single copy that was limited to GR5 (Fig. 2).

Screening of *C. rosea* strains for the GR5-specific marker. The SCAR primer pair was tested for specificity against the 14 strains plus 68 additional strains of *C. rosea* and *Gliocladium* spp. (Table 1; Fig. 3). Only DNA from the GR5 strain could be amplified using the SCAR primers to produce the diagnostic product. We therefore hypothesize that this sequence is unique for GR5. Under suboptimal reaction conditions, DNAs from two other strains (GJS 89-34 and GR47 [Table 1]) could be amplified with the SCAR primers, but both strains could be differentiated from GR5 by UP-PCR (Fig. 4). The fragments, from these strains were sequenced, and they differed slightly from each other and from the GR5 sequence (96 to 99% similarity).

Detection of GR5 in soil. DNA was extracted from GR5-inoculated and control field soils from 31 different fields,

TABLE 3. Soil samples

Soil sample(s)	Origin
B1DK, B5DK, B6DK, B7DK, B11DK, B12DK, B13DK, B14DK, B16DK, B17DK, B22DK, B23DK, B24DK	Barley fields, DK[a]
W3DK, W4DK, W15DK, W18DK, W20DK, W21DK	Wheat fields, DK
S9DK	Sugar beet fields, DK
G2DK	Grass field, DK
R10DK	Rye field, DK
R19DK	Rape, DK
25GR	Leros, Greece
26AU	Mudge, Australia
B2aRU, B2bRU, B2cRU, B2dRU	Barley fields, Russia
O1RU, O3RU	Oat fields, Russia

[a] DK, Denmark.

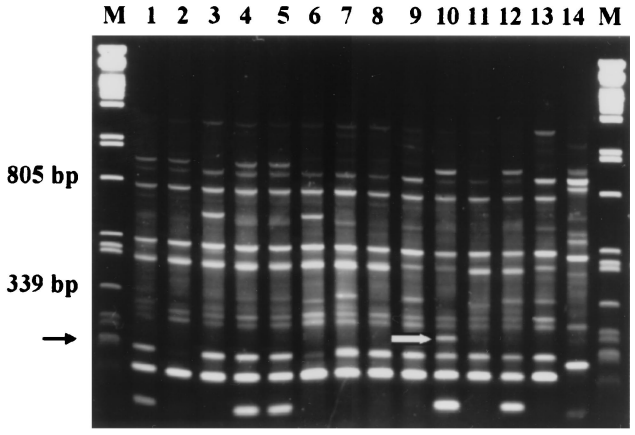

FIG. 1. UP-PCR banding profiles for *C. rosea* strains generated with the AS15inv-AA2M2 UP primer combination. Lanes 1 to 14, strains GR3, IK726, GR6, GR10, GR12, GR11, GR9, GR8, GR4, GR5, GR1, GR13, GR7, and GR2, respectively. Lanes M, molecular size markers (λ phage DNA digested with *Pst*I). Arrows shows the marker of interest for strain GR5.

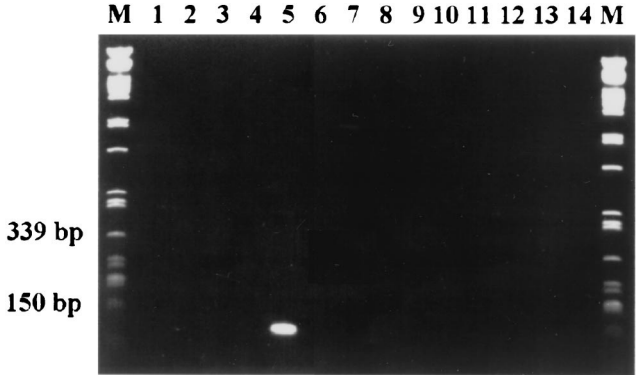

FIG. 3. Testing of *C. rosea* strains for the AS15inv-AA2M2 marker using the SCAR primer set. Lanes 1 to 14, strains IK726, GR12, GR10, GR13, GR5, GR3, GR2, GR4, GR7, GR6, GR11, GR9, GR8, and GR1, respectively. Lanes M, molecular size markers (λ phage DNA digested with *Pst*I).

mostly Danish (Table 3), and tested in dilution series in PCR using primers that amplify the ITS1 region of fungal nuclear ribosomal DNA (positive control for amplification), followed by testing with the SCAR primer pair. GR5 was isolated in 1991 from a field neighboring soil B24DK. In some DNA extractions from noninoculated B24DK, a weak product of similar size was produced using the SCAR primers. This might indicate that GR5 or strains carrying the GR5-specific SCAR marker were naturally present in that soil at a low level. In all other field soils tested, no background level of the GR5 marker was detected. For the two GR5-inoculated soils we could detect GR5 using the SCAR primer pair (Fig. 5).

We tested two of the noninoculated soils for the presence of indigenous *C. rosea* strains by plate diluting on semiselective media. We found that soil B23DK (Table 3) contained *C. rosea* at a level of approximately 4×10^3 CFU/g (dry weight) of soil and that soil B24DK contained *C. rosea* at a level of approximately 10^3 CFU/g of soil. Representative isolates from the plate dilutions were screened for the GR5-specific marker using the SCAR primers, and all were negative.

DISCUSSION

We have developed a strain-specific PCR-based detection tool for an antagonistic *C. rosea* strain, GR5. This strain can control disease caused by *F. culmorum* efficiently in an in planta bioassay that has been shown to correlate well with field performance (12, 14, 26). The detection assay will be used to detect and monitor GR5 deliberately released into field soil. We used UP-PCR to identify a fragment from which SCAR primers were developed and used for PCR detection of the strain. Markers such as the one we have developed may be important for detection of other biocontrol agents or specific pathogenic microorganisms or for protection of commercial (patent) strains. In general, UP-PCR-derived markers have little, if any, similarity to sequences in known databases, and this 250-bp marker also lacked any significant sequence similarity.

In previous studies, specific primers have been developed for fungal subspecies, varieties, and even strains using ribosomal DNA variable regions (4, 5, 22). Strain differentiation problems often occur as the sample size increases, however, since only a few strains have been used for the primer design in many of the reported examples (see, e.g., references 4 and 15). Similar work has been done with the RAPD technique. Zimand et al. (33) used a set of nine RAPD primers to differentiate

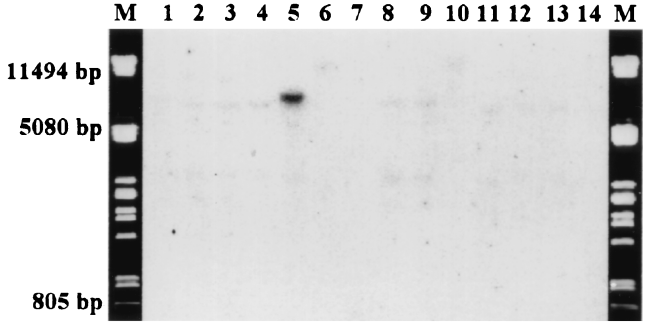

FIG. 2. Southern blot hybridization analysis of the GR5 AS15inv-AA2M2 marker. Lanes 1 to 14, strains IK726, GR12, GR10, GR13, GR5, GR3, GR2, GR4, GR7, GR6, GR11, GR9, GR8, and GR1, respectively. Lanes M, molecular size markers. α-^{32}P-labeled AS15inv-AA2M2 PCR product from strain GR5 was used as the probe.

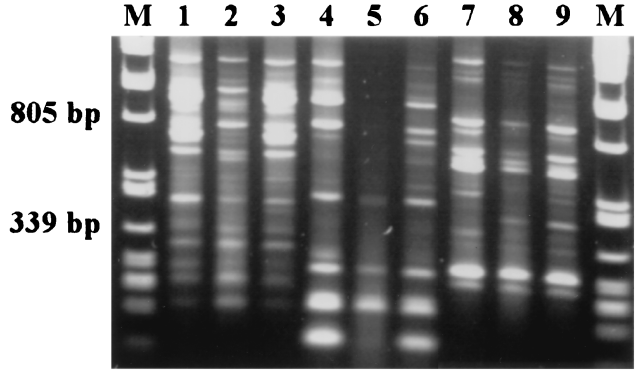

FIG. 4. Differentiation of GR5 from GR47 and GJS 89-34 by UP-PCR. Lanes 1 to 3, profiles generated with UP primer AS15inv. Lanes 4 to 6, profiles generated with UP primer combination AS15inv-AA2M2. Lanes 7 to 9, profiles generated with UP primer AA2M2. Lanes 1, 4, and 7, strain GR5. Lanes 2, 5, and 8, strain GJS 89-34. Lanes 3, 6, and 9, strain GR47. Lanes M, molecular size markers (λ phage DNA digested with *Pst*I).

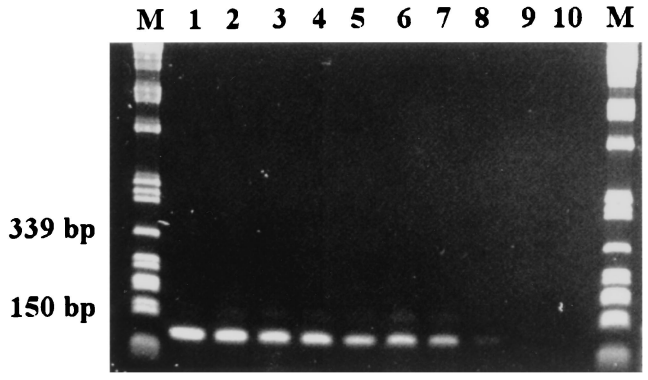

FIG. 5. GR5 detection in the B23DK soil using the SCAR primer set. Lane 1, GR5 (positive control); lanes 2 to 5, soil sample with large amount of GR5 (0.1, 0.01, 0.001, and 0.0001 μl of eluted DNA, respectively); lanes 6 to 8, soil sample with a smaller amount of GR5 (0.1, 0.01, and 0.001 μl of eluted DNA, respectively); lanes 9 and 10, noninfested soil sample (negative control) (0.1 and 0.01 μl of eluted DNA, respectively). Lanes M, molecular size markers.

Trichoderma harzianum strain T-39 from other *Trichoderma* strains, and Abbasi et al. (1) used three RAPD markers to differentiate *Trichoderma hamatum* strain 382 from 45 other *T. hamatum* strains. Abbasi et al. (1) converted the RAPD markers into SCAR markers, none of which was unique for the strain. However, their approach was not tested with noninoculated compost. Abbasi et al. (1) used 180 RAPD primers to screen their *T. hamatum* strains in a strategy similar to ours, but we only used 7 different UP primers in this work.

The specificity of the SCAR primers was tested on 77 *C. rosea* strains and 5 strains from two closely related species, but only GR5 carried this sequence. Of the two strains that responded to the SCAR primers under suboptimal reaction conditions, one was from Guyana and the other was from Denmark. The fragments amplified from these strains were quite similar (96 to 99% similarity) to the GR5 sequence. Although the marker is unique for GR5 among the strains tested, DNA with a similar but slightly varying sequence composition does appear to occur in other *C. rosea* strains.

We also tested the specificity of the primers on DNA in field soil samples obtained from 31 different locations. One of the samples came from the same locality from which GR5 originated (B24DK). Very weak amplification sometimes occurred, indicating the presence of a background level of GR5 or strains with the GR5 marker in this soil. Thus, in order to use the marker as a tool for monitoring GR5 in different soils, it is essential to define the background level of the marker. In the other 30 soils no background was detected, even though at least some of these soils contained indigenous *C. rosea*. The isolates recovered by a plate dilution technique from these soils did not respond to the primers. Detection of a small number of target organisms in an environment requires a high level of sensitivity (19, 23). The assay we have developed could be used to detect GR5 deliberately released into field soil. However, the assay is qualitative, and future work will be devoted to developing a sensitive quantitative assay for facilitating strain counts. This assay will enable us to take a background level of the marker into account in comparative studies and give us the opportunity to identify optimal levels of GR5 in relation to biocontrol efficacy and to study long-term survival after field release. Because mixtures of different strains for biocontrol may enhance the performance of a commercial product, we also are developing similar detection schemes for other antagonistic *C. rosea* strains.

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